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(54) Title: METHOD FOR THE EX–VIVO EXPANSION OF HEMATOPOIETIC STEM CELLS

(57) Abstract

The present invention provides a method for the ex–vivo expansion of hematopoietic stem cells and a culture medium for stem cells.

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METHOD FOR THE EX-VIVO EXPANSION OF HEMATOPOIETIC STEM CELLS

A large number of onco-hematologic diseases can often be treated only with bone-marrow transplantation. It is rather difficult to find a donor with identical HLA within the patient's family and thus, hematopoietic stem cell transplantation from non-related donors represents a novel therapy for those patients who need bone-marrow transplantation but do not have donor-siblings with identical HLA.

Even though the number of bone-marrow donors has increased in donors' registries, only 20-30% candidates find compatible donors, whereas ethnic minorities are poorly represented (Sonnenberg F. et al.., Blood 74:2569-79, 1989).

A severe graft versus host disease (GVDH) is associated with almost 40% transplants of bone marrow obtained from a related donor and with 60-80% transplants based on non-related donors (Hows J et al.. Blood; 68:1322-4, 1986).

Cord blood hematopoietic stem cells can reconstitute the human hematopoietic system and thus they represent an alternative to bone marrow stem cells. The following advantages are associated with stem cells available from placental blood (stored in cord blood banks as cryopreserved cell collections), in comparison with bone marrows available from donor registries,:

1. A good amount of placental blood can be easily obtained, without any risk either for the mother or for the newborn,

2. Infective agents such as cytomegalovirus (CMV) are less frequent in newborns than in adults;

3. Placental blood can be cryopreserved for many years and it is available in short time;

4. a higher possibility to find a donor also for ethnic minorities;

5. less stringent requirements of HLA match (histocompatibility);

6. a less frequent graft versus host disease (GVDH) after transplantation with placental blood (Broxmeyer HE et al.., Int. J. Cell. Cloning 8: 76-91, 1990; Broxmeyer HE et al.., Proc. Natl. Acad. Sci. USA 86:3828-32, 1989).

So far, the use of cord blood in adult patients has encountered some problems due to the content of stem cells in a cord blood unit, which is frequently not sufficient for transplantation in adults (Kurtzberg J. et al.., N. Eng. J. Med. 335:157-166, 1996; Wagner JE et al.., Lancet 346:214-219, 1995; Locatelli F. et al.., Bone Marrow Transplant 18:1095-1101, 1996).

The high doses of chemotherapy, followed by transplantation of stem cells, provoke immunodepression in the patient who thus needs long periods of time for restoring the platelet and erythrocyte activities. Furthermore, the patient needs many transfusions of several units of platelet and erythrocyte blood components, thus increasing the costs of bone marrow transplantation. The ex-vivo expansion of hematopoietic cells may play an important role in the solution of the problems associated with transplantation and its costs. Clinical trials have shown that expanded cells do not

induce toxicity but, on the contrary, they offer several advantages (McAdams TA et al.., Trends Biotechnol. 14:388-96, 1996). The expansion of stem cells from placental blood might improve the therapeutic approach to bone-marrow transplantation for the following reasons:

1. The ex-vivo expansion of hematopoietic precursors maintaining a non-committed population, allows
   a) the transplantation of adult patients (Kurtzberg J et al.. N Engl J Med 335:157-166, 1996; Wagner JE et al.. Lancet 346:214-219, 1995; Locatelli F et al.. Bone Marrow Transplant 17:31-37, 1996; Locatelli F et al.. Bone Marrow Transplant 18:1095-1101, 1996)
   b) the preparation of aliquots which can be frozen in case of relapse of the same patient (Lu L. et al.. In vivo 10:229-32, 1996)
   c) protocols of gene therapy.
2. The ex-vivo expansion of hematopoietic precursors towards a committed population (megakaryocytes and erythroblasts) may provide a cell pool which can be frozen and transplanted into the same patient during the succeeding phases of the disease. (Lu L. et al.. In vivo 10:229-32, 1996). This procedure is in order to shorten the time to hematological reconstitution (Emerson SG. Blood 87: 3082-8, 1996).

The ex-vivo expansion is an interesting approach for maintaining hematopoietic stem cells both qualitatively and quantitatively. The cell maturation obtained through the expansion represents a crucial

point for the achievement of a suitable clinical application of the product. Actually, the expansion effectiveness and the maturation of the expanded cells can be influenced by many factors among which the static or bioreactor culture, the growth factors and the incubation period are the most critical ones (Haylock DN. et al.., Blood 80:1405-12, 1992; Brugger W. et al.., Blood 81:2579-84, 1993; Sato N. et al.. Blood 82:3600-9, 1993). Proliferation and maturation of hematopoietic cells are strictly regulated by factors that positively modulate primitive stem cell proliferation and multilineage or unilineage differentiation. A number of cytokines have been utilized in an attempt to promote human hematopoietic stem cell self-renewal. Although a variety of culture conditions have been defined which promote expansion of committed progenitor cells, conditions do not presently exist that permit long-term expansion of stem cells (Zijlmans JMJ et al.. Proc Natl Acad Sci USA 92:8901-5, 1995; Zijlmans JMJ et al.. Proc Natl Acad Sci USA 95:725-9, 1998; Bradford GB et al.. Exp Hematol 25:445-53, 1997; Rebel VI et al.. Blood 83: 128-36, 1994).

In fact several authors have obtained committed populations: Haylock et al.. achieved a 66-fold expansion using 6 growth factors (IL-1, IL-3, IL-6, G-CSF, SCF) and a 14-day liquid culture. Brugger et al.. obtained a 190-fold expansion with 6 factors (IL-3, IL-1, IL-6, SCF, EPO, interferon-g) in 12-14 days.

So far, no scientific investigation has furnished either the final evidence of the CD34+ stem-cell self renewal ability or a procedure through which such non-

committed population could be maintained. The loss of population with CD34+ immunophenotype in favour of subpopulations with CD34+/33+ and CD34+/38+ phenotype, i.e. the continuous decrease of precursors, unambiguously shows the loss of proliferative ability of the expanded precursors. Up to now, this has limited the use of expanded-cell suspensions for transplantation, in all those cases in which hematopoiesis was not supported by sources of non-purified and non-expanded stem cells.

So far, IL-3 and SCF are the most used cytokines for obtaining a precursor expansion, even if the addition of these growth factors has a negative effect, because they induce maturation of primitive stem cells (e.g. towards the neutrophil subpopulation) at the expense of self-renewal.

Two cytokines named Flt-3 and TPO have recently been found and more recently they have been deeply investigated in combination with other growth factors (Koller MR et al..., J. Hematother. 5:449-59, 1996; Kaushansky K. et al..., Nature, 369:519-20, 1995). It has been reported that c-mpl is expressed exclusively on hematopoietic tissue, particularly on CD34+ hematopoietic stem cells, megakaryocytes and platelets. Even if the role of TPO and Flt-3 is becoming increasingly evident on the normal haematopoieis (Kaushansky K et al... Blood 86:419-31, 1995). These cytokines have been found not to have a high activity when used alone, but when combined with other cytokines, their real effect cannot be appreciated due to the presence of more potent growth factors such as IL-3 and SCF.

The CD34+38- immunophenotype defines a primitive subpopulation of progenitor cells and the maintenance of these cells continues to be a subject of ongoing study (randal TD et al.. Blood 87:4057, 1996).

Now, a method has been found for the ex-vivo expansion of stem cells which allows to maintain a part of the cell population in form of CD34+ non-committed cells having self-renewal properties. This method allows to maintain the expansion in constant proliferative growth up to 15 weeks or more, thus obtaining the desired qualitative (CD34+/38- phenotype) and quantitative (cell number) expansion.

The method according to the invention comprises culturing stem cells in a culture medium containing a mixture of cytokines/growth factors consisting of thrombopoietin (TPO), interleukin 6, interleukin 11 (IL-6 and IL-11) and flt-3 ligand (FL).

These components are commercially available or they can be prepared through recombinant-DNA techniques, and they are added to the culture medium at a final concentration of 1 to 100 ng/ml. Preferably, each of these components is added to the culture medium at a final concentration of 10 ng/ml. The culture medium, which is a further object of the invention, contains also other conventional components such as fetal bovine serum or albumin. The culture of stem cells, which can be obtained, for example, from cord blood or from other sources (e.g. bone-marrow or peripheral blood), is carried out under humidified atmosphere containing 5% CO_2 for periods of time ranging from few days (for example 10 days) to several weeks (20 or more). The

expansion obtained by the method of the invention is clearly higher than that so far obtainable with different cytokine cocktails. In fact recently a combination of cytokines including FL+IL-6+IL-11 has been tested in a paper of Ohmizono et al.. (Leukemia 11: 524-30, 1997), but the efficiency of the expansion has been lower and for up to 21 days.

Description of figures:

Figure 1 shows the cellular expansion obtained using the method and the medium of the invention, therefore after culture of purified CD34+ cells in the presence of serum-free medium and FL+TPO+IL-6+IL-11. The Cd34+ cells were purified through separation columns and cultured for 15 weeks in serum-free medium and FL+TPO+IL-6+IL-11. Every week the cultures were demipopulated and cytokines and fresh medium were added. From the data reported in figure 1, a 10^7-fold expansion of the number of nucleated cells is observed and it is clear that an expansion higher than 10^6 can be obtained just after 15-weeks culture.

Figure 2 shows the median of the expansion of CD34+/38- cells obtained after culture in the serum free medium containing FL+TPO+IL-6+IL-11. The cells were stained after expansion and counted by flow cytometric analysis using a FACScan analyzer. This figure shows that the number of CD34+/CD38- non-committed cells is 100.000 times higher than the initial number.

Figure 3 shows the median of expansion of CFU-GM colonies obtained in presence of serum-free medium containing FL+TPO+IL-6+IL-11. The cells were seeded in methylcellulose after expansion. This figure shows that

the number of CFU-GM colonies is 100.000 times higher than the initial number.

The following examples illustrate the invention in more detail.

EXAMPLE 1

CD34+ cell purification

Cord Blood samples were collected after cesarean section or vaginal delivery. Cord blood was collected by venipuncturing the main vessel at the free end of the cord using a Maco Pharma bag with 29 ml citrate-phosphate-dextrose (CPD) as anticoagulant. The placenta was washed out to collect the blood that remains in vessels. Mononuclear cells were isolated by Ficoll gradient (1.077 g/ml; Lympholite-H, Cedarlane Laboratories, Ontario, Canada) and CD34+ cells were purified through separation columns (CellPro Inc, Bothell, Wash., USA or MiniMACS, Milteryi, Germany).

EXAMPLE 2

An aliquot of the CD34+ target cell fraction was analyzed to determine purity by flow cytometry. The final recovery of CD34+ cells ranged from 70% to 98% of the initial CD34+ population and the analysis of the enriched cell fraction, performed with an anti-CD34+ monoclonal antibody (Becton Dickinson, Mountain View, California, USA) revealed a purity of 85% to 98% CD34+ cells. The trypan-blue dye exclusion test showed a viability of 96%-99%. The CD34+ cells were seeded for the clonogenic assay.

Liquid cultures of CD34+ cells

CD34+ cells were plated at 3-5x10^4/ml, in Tissue Culture Flasks. A serum-free medium containing different

concentrations of growth factors as indicated was used.

TPO, FL, IL-6 and IL-11 (Peprotech EC Ltd, London , England) were added to stroma-free liquid cultures of purified CD34+ CB cells. Cells were incubated for more than 15 weeks at 37°C in fully humidified atmosphere in 5% CO_2 air.

Clonogenic assay

Colony-forming units (CFU) were evaluated in 35 mm dishes by plating $2.5x10^3$ nucleated cells in 1 ml medium containing 0.9% methylcellulose, 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 3 U/ml erythropoietin, 50 ng/ml SCF, 10 ng/ml GM-CSF, 10 ng/ml IL-3 (StemCell Technologies, Vancouver, Canada). After 14 days of culture at 37°C in a 5% CO_2 fully humidified atmosphere, cultures larger than 50 cells were scored by microscopy as colony forming cells (CFC), i.e. the sum of CFU-GM (containing granulocytes and macrophages), Burst Forming Unit-Erythroid (BFU-E, containing erythroid cells), and CFU-GEMM (containing myeloid cells, erythroid cells and megakaryocytes).

Flow Cytometry

CD34+ cultured cells were stained with one or more of the following monoclonal antibodies: anti-CD34, -CD61 (gpIIIa), -CD38. For flow cytometry analysis $5x10^5$ cells were incubated with monoclonal antibodies for 30 minutes at 4°C and washed twice in PBS. Cells were analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson) equipped with a filter set for FITC-PE dual-color fluorescence.

The percent of stained cells was determined as compared to PE- and FITC-conjugated mouse IgG1 isotypic

control (Becton Dickinson). Cell viability was evaluated by staining cells with 7-AAD, and viable cells were gated.

In vitro assay in NOD/SCID mice

The use of the ex vivo expanded cells in NOD/SCID mice is up to now an experiment to demonstrate the capacity of these expanded cells to reconstitute the bone marrow with a complete engraftment. Moreover these experiments add a new step towards the implementation of clinical expansion protocols.

The NOD/SCID mice were sublethally irradiated immediately before intravenous tail-vein injection containing an appropriate number of expanded cells. The duration of this assay was 8 weeks. Flow cytometric analysis was performed on peripheral blood and bone marrow after death of mice. The presence of cells positive for human CD34+, CD19+, CD42a+ antigens was relevant after transplantation (until to 15-19%, 14-16%, 13-15% of the total cells respectively). All colonies, obtained after culturing bone marrow cells (clonogenic assay), were plucked from plates and were analyzed by a human-specific PCR (Polymerase Chain Reaction): the PCR signals were positive for the human Cart-1 gene. Controls consisted of PCRs for human Cart-1 from human peripheral blood leukocytes (positive) and normal mouse bone marrow cells (negative). The signals were of human origin.

This assay has indicated the capacity of these expanded cells to home the mice bone marrows.

CLAIMS

1. A method for the ex vivo expansion of hematopoietic stem cells which comprises culturing said stem cells in a culture medium containing a mixture of cytokines/growth factors consisting of thrombopoietin, interleukin 6, interleukin 11 and Flt-3 ligand.

2. A method according to claim 1, characterized in that the hematopoietic stem cells are obtained from cord blood or other suitable sources.

3. A method according to claim 1 or 2, characterized in that the culture is maintained for a time ranging from 10 days to 15 weeks.

4. A method according to any one of the above claims, in which the culture medium contains interleukin 6, interleukin 11, thrombopoietin and Flt-3 ligand, each at a concentration ranging from 1 to 100 ng/ml.

5. A method according to claim 4, in which the concentration of interleukin 6, interleukin 11, thrombopoietin and Flt-3 ligand in the culture medium is 10 ng/ml.

6. A method according to claim 1 wherein the cells are 1000000-fold expanded.

7. A culture medium for hematopoietic stem cells containing a mixture of cytokines/growth factors consisting of thrombopoietin, interleukin 6, interleukin 11 and Flt-3 ligand.

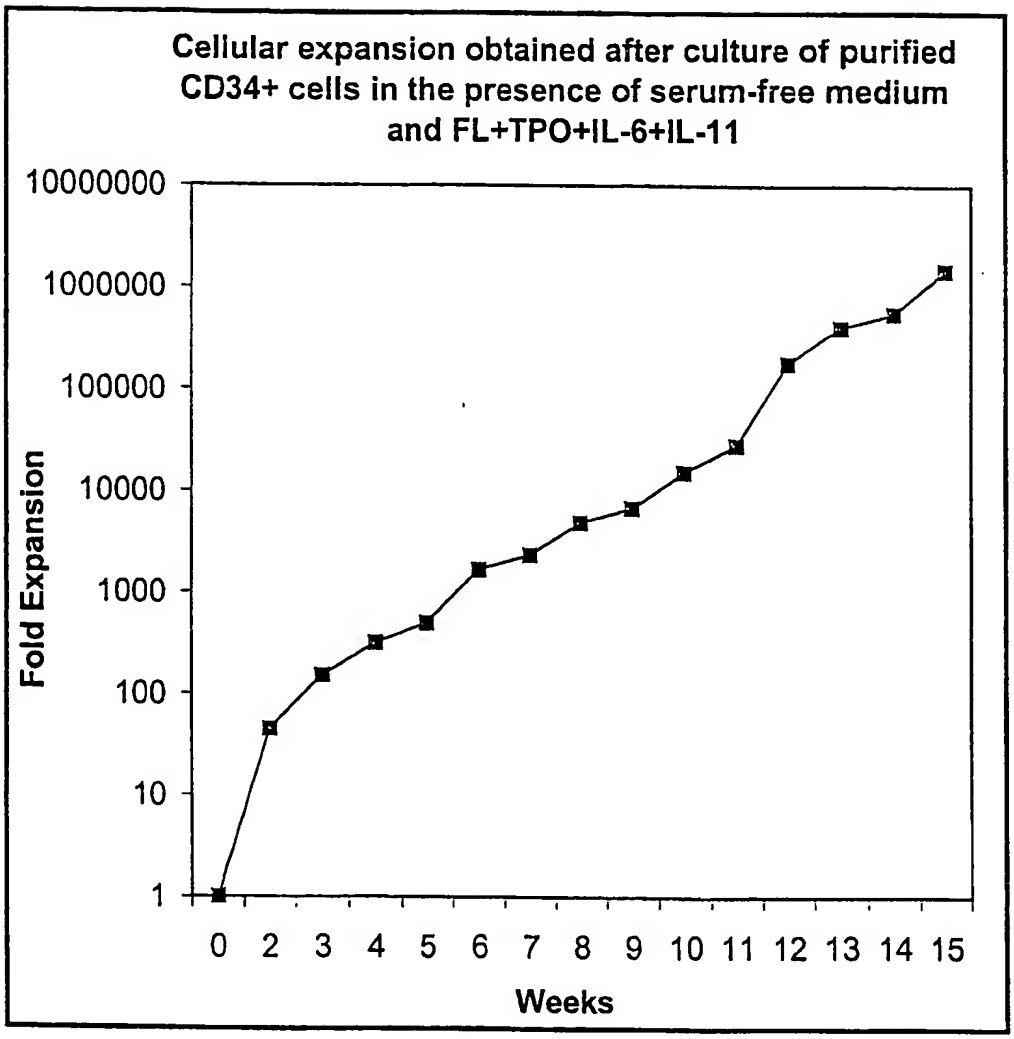
8. A culture of hematopoietic stem cells obtainable by the method of claims 1-6.

9. A colture according to claim 8 in which the number of CD34+/CD38- non-committed cells is 100.000

times higher than the initial number.

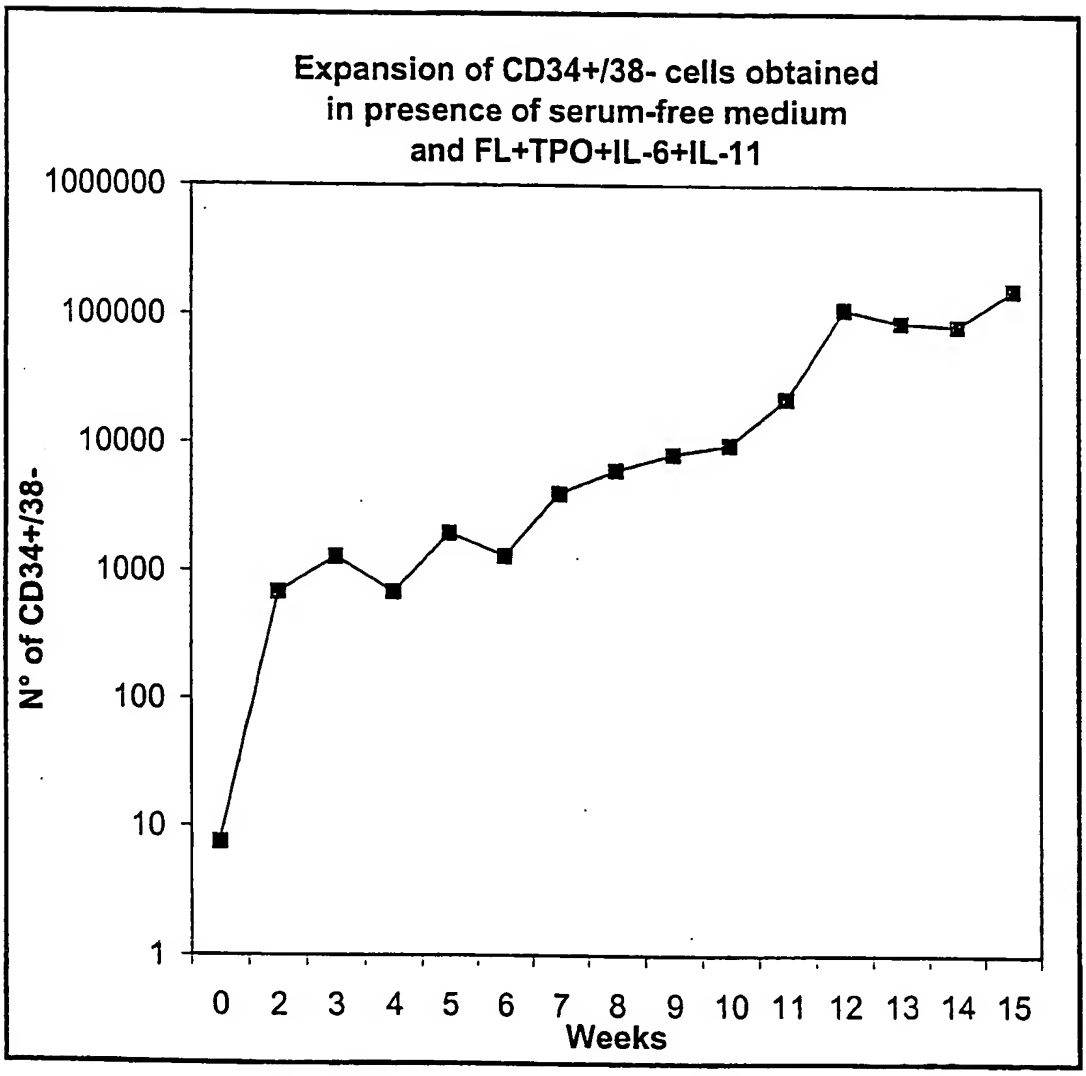
SHEET 1/3

FIG.1



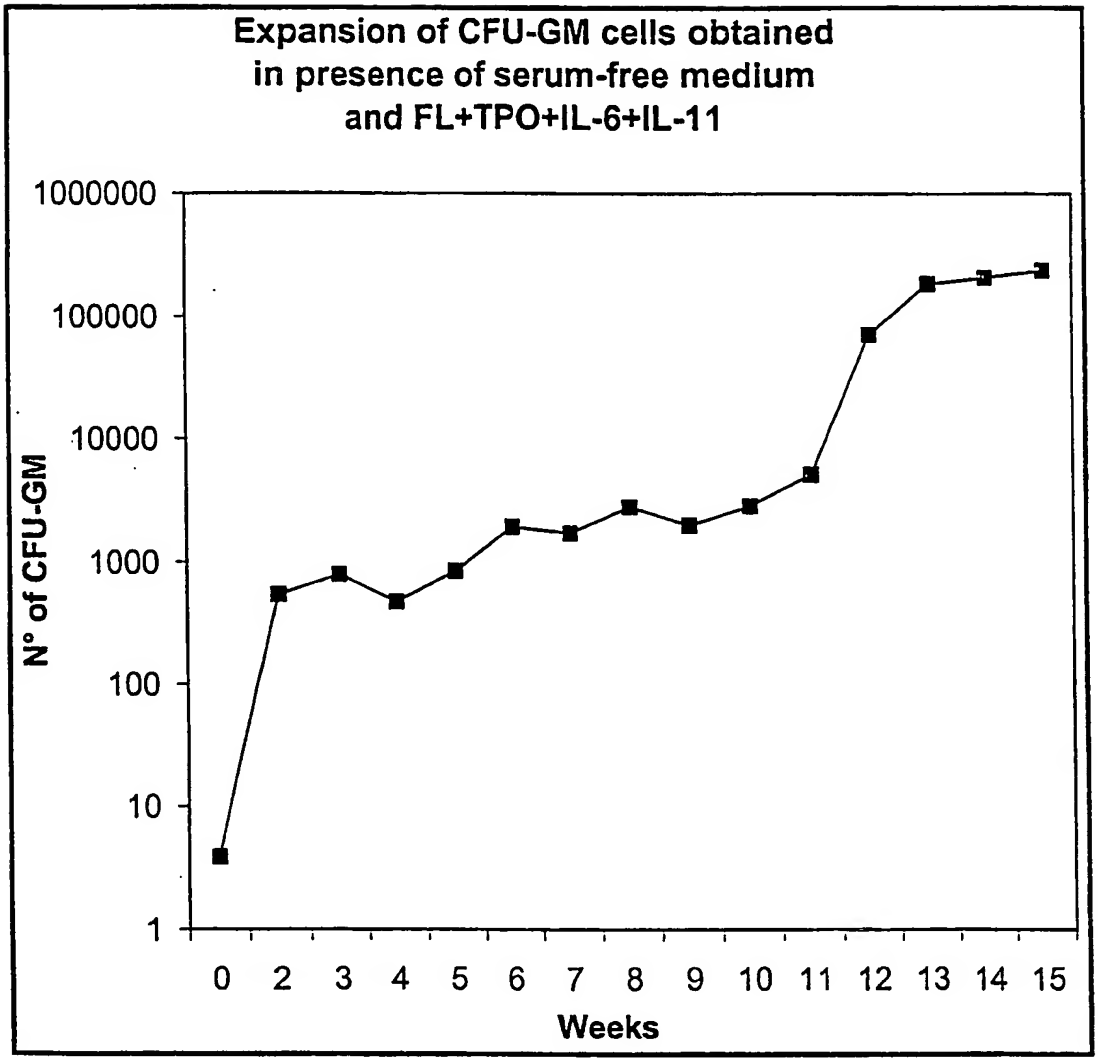
SHEET 2/3

FIG. 2



SHEET 3/3

FIG. 3



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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OHMIZONO Y. ET AL: "Thrombopoietin augments ex vivo expansion of human cord blood-derived hematopoietic progenitors in combination with stem cell factor and flt3 ligand" LEUKEMIA, vol. 11, no. 4, April 1997, pages 524-530, XP002053902 *whole article, especially table 2* ---	1-9
X	WO 97 16535 A (SANDOZ LTD ;SYSTEMIX INC (US); SANDOZ AG (DE); SANDOZ AG (AT)) 9 May 1997 see the whole document ---	8,9
A	EP 0 627 487 A (IMMUNEX CORP) 7 December 1994 see the whole document -----	1-9

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Information on patent family members

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